The invention relates to a process for increasing the level of differentiation and/or proliferation of skin fibroblasts and/or for increasing the level of differentiation of skin keratinocytes by applying to the skin a composition comprising an effective amount of ascorbic acid or of at least one of its analogues.

The invention also relates to a process for stimulating the synthesis of cutaneous vimentin by applying to the skin a composition comprising an effective amount of ascorbic acid or of at least one of its analogues. The invention also relates to a process for stimulating the synthesis of cutaneous keratin 10 by applying to the skin a composition comprising an effective amount of ascorbic acid or of at least one of its analogues.

Human skin consists of two compartments, namely a superficial compartment, the epidermis, and a deep compartment, the dermis.

Natural human epidermis is composed mainly of three types of cells: keratinocytes, which form the great majority, melanocytes and Langerhans cells. Each of these cell types contributes, by virtue of its intrinsic functions, towards the essential role played in the body by the skin, in particular the role of protecting the body against external attack (climate, ultraviolet rays, tobacco, etc.), which is referred to

as the "barrier function". Poor renewal of these cells, and more particularly of the keratinocytes, which is observed in particular with age, leads to poor protection of the skin, which then acquires a dry and/or dull appearance.

The dermis gives the epidermis a solid support. It is also the epidermis' nourishing factor. It consists mainly of fibroblasts and of an extracellular matrix which is itself composed mainly of collagen, elastin and a substance known as ground substance, these components being synthesized by the fibroblasts. Leukocytes, mastocytes and tissue macrophages are also found therein. It also contains blood vessels and nerve fibres.

Vimentin fibres are found extensively in the dermis, since they correspond to the intermediary filament of fibroblasts. These vimentin fibres are also present in melanocytes and in Langerhans cells of the epidermis, and can also be present in keratinocytes when they are in a hyperproliferative state.

Keratins are the intermediary filaments of epithelial cells, such as the keratinocytes in the skin. Thus, four types of keratin exist in the epidermis, including keratin 10, referred to as K10, which is specific for the state of differentiation of the keratinocytes.

With age, the quality of the skin diminishes, and in particular thinning of the dermis is observed. It is also accepted that extrinsic factors such as ultraviolet rays, tobacco or certain treatments (glucocorticoids, vitamin D and derivatives, for example) also have a negative effect on the skin.

The importance of cell renewal and the quality of this renewal can thus be appreciated, both at the epidermal level and at the dermal level, in order thus to be able to combat extrinsic attack which damages the skin, in particular by reducing its barrier function, and to combat the signs of ageing of the skin, whether this is chronobiological or light-induced ageing.

One of the aims of the present invention is thus to increase the level of differentiation and/or proliferation of skin fibroblasts and/or to increase the level of differentiation of skin keratinocytes in order thus to combat extrinsic attack, whether this is physical or chemical attack, which damages the skin, in particular by reducing its barrier function, and to combat ageing of the skin, whether this is chronobiological or light-induced ageing.

The Applicant has now discovered that ascorbic acid applied topically to the skin increases the level of differentiation and/or proliferation of skin fibroblasts and/or increases the level of

differentiation of skin keratinocytes, by applying to the skin a composition comprising an effective amount of ascorbic acid or at least one of its analogues.

Ascorbic acid (or vitamin C) is known to stimulate collagen synthesis, by preventing, in the capacity of a co-factor, the self-inactivation of lysine hydroxylase and proline hydroxylase enzymes and by increasing the synthesis of procollagen mRNAs. Ascorbic acid (or vitamin C) is also known to stimulate the synthesis of skin elastin. Mention may be made in this respect of US patents 5 801 192 and 4 983 382 and patent EP 0 717 983. Mention may also be made of an article entitled "Pola to incorporate vitamin C in new cosmetics line for skin care" in the Japan Economic Journal of 5 June 1984 (page 15). Thus, it has been described that ascorbic acid used in cosmetic compositions makes it possible in particular to treat wrinkles (Fragrance Journal, Vol. 8, No. 6(45) (1980) pp. 38-43, "Cosmetic and vitamin - action and safety to dermatology").

One subject of the invention is thus the use of an effective amount of ascorbic acid or of one of its analogues in a composition or in the preparation of a composition intended to be applied to the skin to increase the level of differentiation and/or proliferation of skin fibroblasts and/or to increase the level of differentiation of skin keratinocytes.

A second subject of the invention is the use of an effective amount of ascorbic acid or of one of its analogues in a composition or in the preparation of a composition intended to be applied to the skin to stimulate the synthesis of cutaneous vimentin.

A third subject of the invention is the use of an effective amount of ascorbic acid or of one of its analogues in a composition or in the preparation of a composition intended to be applied to the skin to stimulate the synthesis of cutaneous keratin 10.

A subject of the invention is also a process for increasing the level of differentiation and/or proliferation of skin fibroblasts in an individual with an abnormally low level of fibroblast differentiation and/or proliferation, comprising the topical application to the skin of an effective amount of ascorbic acid or of one of its analogues.

A subject of the invention is also a process for increasing the level of differentiation of skin keratinocytes in an individual with an abnormally low level of keratinocyte differentiation, comprising the topical application to the skin of an effective amount of ascorbic acid or of one of its analogues.

Specifically, the Applicant has discovered that ascorbic acid applied topically to the skin increases the synthesis of vimentin mRNA and thus increases the level of vimentin synthesis. It has also

discovered that ascorbic acid applied topically to the skin increases the synthesis of keratin 10 mRNA and thus increases the level of synthesis of keratin 10.

These proteins, which are intermediary filaments of skin cells, are thus representative of the proliferative and/or differentiating state of skin cells, more particularly of the cells of the dermis and the epidermis. More particularly, vimentin, which is the intermediary filament of fibroblasts, is representative of the proliferative and/or differentiating state of fibroblasts, and keratin 10 is representative of the differentiating state of keratinocytes.

Thus, by topical application of an effective amount of ascorbic acid or its analogues, these skin cells are renewed more rapidly, the appearance of the skin is improved, the skin is more radiant, less dull, firmer, has better tone and is more elastic, wrinkles are attenuated or their appearance is delayed, and the signs of ageing of the skin are diminished.

Advantageously, the ratio of the synthesis of vimentin mRNAs to that of keratin 10 due to the topical application of ascorbic acid is comparable to that without topical application of ascorbic acid. This indicates that the state of the skin, after topical application of ascorbic acid, is maintained in a normal state (without, for example, hyperproliferation or

hyperdifferentiation of one dermal or epidermal compartment relative to the other).

The ascorbic acid analogues are, more particularly, its salts, especially such as sodium ascorbate and magnesium or sodium ascorbylphosphate, its esters, especially such as its acetic, propionic or palmitic esters, or its sugars, especially such as glycosyl ascorbic acid.

The ascorbic acid is generally in L form, since it is usually extracted from natural products.

The effective amount of ascorbic acid or its analogues which can be used according to the invention is, of course, that which is necessary to obtain the expected effects according to the invention. To give an order of magnitude, this amount preferably represents from 0.001% to 20% of the total weight of the composition, preferably from 0.1% to 15% of the total weight of the composition and advantageously from 3% to 10% of the total weight of the composition.

In addition, the composition of the invention is used for a period which is sufficient to obtain the expected effects according to the invention. To given an order of magnitude, this duration can be a minimum of 15 days, but can also be more than 4 weeks, or even more than 8 weeks.

The composition of the invention which is intended for topical application contains a

physiologically acceptable medium, that is to say a medium which is compatible with the skin, including the scalp, mucous membranes and/or the eyes, and can in particular constitute a cosmetic or dermatological composition.

This composition can be in any pharmaceutical form normally used in cosmetics and dermatology, and it can especially be in the form of an aqueous solution which may be in gelled form, a dispersion of the lotion type which may be in two-phase form, an emulsion obtained by dispersing a fatty phase in an aqueous phase (O/W) or, conversely, (W/O), or a triple emulsion (W/O/W or O/W/O) or a vesicular dispersion of ionic and/or nonionic type. These compositions are prepared according to the usual methods.

The composition of the invention can consist, for example, of a lotion, a gel, a cream or a milk and, for example, a make-up-removing or cleansing lotion or milk, a shampoo or a shower gel.

The example which follows illustrates the invention without limiting it in any way. In the compositions, the proportions indicated are percentages by weight, except where otherwise indicated.

Example:

1. Method

10 women aged between 55 and 60 applied, to the lower neck, once a day for 3 months, on one side a water-in-oil emulsion (vehicle or placebo), and on the other side the same water-in-oil emulsion but also comprising 5% vitamin C (= composition or active agent).

Composition:

L-Ascorbic acid	5.00%
Sodium hydroxide	1.83%
Citric acid monohydrate	1.24%
Disodium EDTA	0.05%
Apricot kernel oil	3.00%
Silicone oil	4%
Cyclopentasiloxane and dimethicone copolyol	20%
Dimethicone and dimethiconol	3%
Glycerol	23%
Propylene glycol	4%
Fillers	7%
Preserving agents	0.30%
Water	qs 100.00%

Biopsies of these treated areas were then taken.

2. Extraction and purification of the total RNAs

The biopsies are ground under liquid nitrogen in a Mikrodismembrator S (Braun). The powder obtained is collected in a Teflon capsule with 2 ml of lysis solution (5M guanidine isothiocyanate, 0.1M mercaptoethanol, 0.017M sodium laurylsucosyl, 0.025M sodium citrate pH7, 3 µl/ml antifoam). The suspension is transferred into a tube and is shaken at room temperature for 15 minutes. The lysate is deposited on the surface of a cushion of 1.4 ml of 5.7M caesium chloride, 0.1M EDTA pH 7 in a 3.8 ml polyallomer tube for an SW60 rotor (Beckman L70M Ultracentrifuge). Ultracentrifugation is carried out at 35,000 rpm for 18 hours at 20°C. The pellet is rinsed with absolute ethanol, centrifuged at 13,000 rpm at 4°C for 10 minutes and dissolved in 100 µl of distilled water.

3. Quantification of the concentration of total RNA and of specific mRNA

The amount of RNA collected from the biopsies is estimated from the optical density of the solution at 260 nm, and is then measured by amplifying the ribosomal 28S RNA by RT-PCR. The specific mRNAs are measured by quantitative RT-PCR on aliquots of the same dilution of total RNA, stored at -80°C until the time of use.

Measurement of the mRNA of keratin 10 (K10) and of vimentin

The oligonucleotide primers specific for the genes studied comprise 24 bases, have an A-T percentage of close to 50 and are chosen on two different exons in order to avoid amplification of any traces of DNA present in the samples. The optimum amplification conditions (temperature and number of cycles) were determined for each of the genes studied, taking into account their level of expression in the skin. The RT-PCR is carried out using the Gene Amp rTth kit from Perkin Elmer or the Titam kit from Boehringer.

Each RT-PCR reaction is carried out in the presence of a known number of copies of a synthetic RNA created in the laboratory, containing the oligonucleotide primer sequences specific for the mRNAs of interest and whose amplification product has a molecular size which distinguishes it from the endogenous mRNA. This multistandard makes it possible to control and calculate the yield for the reverse transcription and the amplification reaction.

The amplification products are analysed by polyacrylamide gel electrophoresis followed by staining with CyberGreen. The intensity of the fluorescent signals is measured using a Fluoro S MultiImager. The results are corrected for the RT-PCR yield and are

expressed in arbitrary units per unit of ribosomal 28S RNA.

4. Statistical analysis

The statistical analysis was carried out using the unilateral Student t Test on the ratios of the Active agent (vitamin C)/Placebo (= A/P) values.

$$t(n-1) = \frac{(M\ A/P - 1)Vn}{M\ \text{standard deviations}}$$

For a degree of freedom n-1 = 9, the A/P ratio is significantly greater than 1 with a probability of greater than 95% for a value of $t > 1.83$ and a probability of greater than 99% for a value of $t > 2.82$.

5. Results

Measurement of the total RNA obtained from the biopsies

The total amount of RNA purified from the biopsies is evaluated in a first stage by measuring the optical density at 260 nm and their quality is estimated by measuring the 260/290 nm OD ratio.

Largely sufficient amounts of RNA were obtained from each of the biopsies (between 2.1 and 6.3 µg) with a satisfactory degree of purity (260/280 OD ratio).

The concentration of total RNA is brought by dilution to a calculated value of 4 nanograms per µl. This process allows the reverse transcription and amplification reactions to be carried out on similar amounts of total RNA for all the samples. The amount of total RNA present in the dilute solution is determined quantitatively by measuring the ribosomal 28S RNA, this being carried out in triplicate.

This same RNA solution will be used for all the measurements of the specific RNAs, the results of which are expressed in 28S RNA units.

<u>Measurement of the equilibrium level of the vimentin and keratin 10 mRNAs</u>

The results expressed in arbitrary units per unit of 28S RNA are detailed in Tables 1 and 2.

Table 1: Vimentin mRNA

Subject	Active agent	Placebo	A/P
a	96.7	51.4	1.88
b	70.9	55.0	1.29
c	67.5	77.7	0.87
d	200.3	131.1	1.53
e	123.0	91.1	1.35
f	106.0	102.5	1.03
g	98.5	92.5	1.06
h	81.4	98.6	0.83
i	112.9	128.6	0.88
j	81.7	66.1	1.24
Average	103.9	89.5	1.20*
Standard deviation	38.4	27.6	0.33

Unilateral Student test: *t = 2.02, P<0.05

Seven out of 10 subjects show an equilibrium level of vimentin mRNA which is increased by ascorbic acid.

Table 2: Keratin 10 (K10) mRNA

Subject	Active agent	Placebo	A/P
a	13.4	8.5	1.58
b	10.3	3.9	2.64
c	12.8	13.8	0.93
d	44.2	48.9	0.90
e	25.5	21.8	1.17
f	40.0	23.8	1.68
g	27.4	14.6	1.88
h	21.6	18.4	1.17
i	35.2	28.7	1.23
j	19.5	13.4	1.46
Average	25.0	19.6	1.46**
Standard deviation	11.8	12.6	0.52

Unilateral Student test: **t = 2.95, $p<0.01$

Eight out of 10 subjects show an equilibrium level of keratin 10 mRNA which is increased with ascorbic acid.

The vimentin/keratin 10 ratio is detailed in Table 3.

Table 3: Vimentin/keratin 10 (VIM/K10) ratio

	VIM / K10	
Subject	Active agent	Placebo
a	7.22	6.05
b	6.88	[14.10]
c	5.27	5.63
d	4.53	2.68
e	4.82	4.18
f	2.65	4.31
g	3.59	6.34
h	3.77	5.36
I	3.21	4.48
j	4.19	4.93
Average	4.61	4.88

These results indicate that the biopsies contain a proportion of keratin 10 mRNA and vimentin mRNA which is comparable on the side treated with ascorbic acid and the placebo. The results also indicate that the biopsies were taken uniformly from the various individuals. The b- placebo sample is outside the norm.

When the vimentin measurements are compared to the equivalent measurements taken for procollagen I or III mRNA, the average value of these ratios calculated for the series of treated samples and placebo samples is very close, indicating a coordinated modulation of procollagen and vimentin expression. In

addition, if it is considered that vimentin is representative of the dermal compartment, since it is the intermediary filament of fibroblasts, then increasing the expression of procollagens I and III is accompanied by a parallel increase in vimentin expression and suggests that ascorbic acid either induces an increase in the number of connective cells in the dermis or induces activation of their biosynthetic phenotype.